EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids (CEF); Scientific Opinion on Flavouring Group Evaluation 201Rev1: 2-Alkylated, aliphatic, acyclic alpha,beta-unsaturated aldehydes and precursors, with or without additional double-bonds, from chemical subgroup 1.1.2 of FGE.19

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SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 201 Revision 1 (FGE.201Rev1):

2-Alkylated, aliphatic, acyclic alpha,beta-unsaturated aldehydes and precursors, with or without additional double-bonds, from chemical subgroup 1.1.2 of FGE.19.[1]

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to consider in this revision 1 of Flavouring Group Evaluation 201, the additional data on genotoxicity submitted by the Industry on two substances, 2-methylpent-2-enal [FL-no: 05.090] and 2 methylcrotonaldehyde [FL-no: 05.095], from subgroup 1.1.2 of FGE.19. First the Panel concluded that genotoxicity data on [FL-no: 05.095] can be representative for the substances [FL-no: 02.174, 05.033, 05.090, 05.105, 05.107 and 05.126], but not for [FL-no: 05.130, 05.178, 09.177 and 09.931], for which it was concluded in the previous version of this FGE that the available data were insufficient to evaluate their genotoxicity. Secondly, the Panel considers that the mutagenicity hazard could not be cleared by the endpoints evaluated in the *in vivo* micronucleus assay submitted. The Panel therefore concluded that further data are required in order to clarify the genotoxic potential of this subgroup. The Panel considers the Comet assay with [FL-no: 05.095] as test material and performed on liver, blood and first site of contact, as a preferred option to further investigate the genotoxicity *in vivo*.

1 On request from the Commission, Question No EFSA-Q-2012-00243, EFSA-Q-2012-00072, EFSA-Q-2012-00071, EFSA-Q-2012-00070, EFSA-Q-2011-01088, EFSA-Q-2011-01087, EFSA-Q-2011-01086, EFSA-Q-2011-01085, EFSA-Q-2011-01084, EFSA-Q-2011-01083, EFSA-Q-2011-01082, adopted on 24 May 2012.

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KEY WORDS

α,β-Unsaturated aldehydes, 2-alkylated substances, flavouring substances, safety evaluation.

SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the Panel) to provide scientific advice to the Commission on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel was asked to evaluate flavouring substances using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000.

In the present revision of FGE.201, FGE.201Rev1, there has been a reassessment of seven substances [FL-no: 02.174, 05.033, 05.090, 05.095, 05.105, 05.107 and 05.126] from subgroup 1.1.2 of FGE.19, due to additional genotoxicity data submitted by Industry.

The Flavouring Group Evaluation 201 (FGE.201), corresponding to subgroup 1.1.2 of FGE.19, concerns eight aliphatic acyclic 2-alkylated α,β-unsaturated aldehydes with or without additional double bonds [FL-no: 05.033, 05.090, 05.095, 05.105, 05.107, 05.126, 05.130 and 05.178] and three precursors for such aldehydes [FL-no: 02.174, 09.177 and 09.931].

In the previous version of this Opinion, FGE.201, the Panel had expressed the following view.

No carcinogenicity studies are available for the eight α,β-unsaturated aldehydes and the α,β-unsaturated aldehydes anticipated to be formed from the precursors in subgroup 1.1.2.

The genotoxicity concern with respect to this group of substances due to the presence of an α,β-unsaturated aldehyde group (or precursor for this) cannot be ruled out based on the genotoxicity data and (quantitative) structure-activity relationship (Q)SAR predictions available.

Therefore, the Panel concluded that a genotoxic potential of the 11 α,β-unsaturated aldehydes and alcohols and related esters in the present FGE.201 could not be ruled out. Accordingly, these 11 substances cannot presently be evaluated through the Procedure. Additional data on genotoxicity on substances representative for this subgroup should be provided according to the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008bb).

In response to the Panel request expressed in FGE.201, the Flavouring Industry has submitted additional genotoxicity data.

Based on these data the Panel concluded that there is some evidence for [FL-no: 05.095] and an indication for [FL-no: 05.090] to show a potency for the induction of gene mutations *in vitro*. Furthermore, the Panel considered that the mutagenicity hazard could not be cleared by the endpoints evaluated in the *in vivo* micronucleus assay and that further data are required in order to clarify the genotoxic potential of this subgroup. A Comet assay performed with [FL-no: 05.095] and performed on liver, blood and first site of contact was considered as a preferred option to further investigate the genotoxicity *in vivo*. Finally, the Panel concluded that the genotoxicity data for [FL-no: 05.095] could be representative for [FL-no: 02.174, 05.033, 05.090, 05.105, 05.107 and 05.126] but not for the remaining substances of this subgroup [FL-no: 05.130, 05.178, 09.177 and 09.931] for which it was already concluded in the previous version of this FGE that the available data were insufficient to evaluate their genotoxicity.

JECFA EVALUATION:

61st meeting: http://whqlibdoc.who.int/publications/2004/924166052X.pdf p289

68th meeting: http://whqlibdoc.who.int/publications/2007/9789241209472_eng.pdf p59

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BACKGROUND

Regulation (EC) No 2232/96 of the European Parliament and the Council (EC, 1996a) lays down a Procedure for the establishment of a list of flavouring substances, the use of which will be authorised to the exclusion of all other flavouring substances in the EU. In application of that Regulation, a Register of flavouring substances used in or on foodstuffs in the Member States was adopted by Commission Decision 1999/217/EC (EC, 1999a), as last amended by Commission Decision 2009/163/EC (EC, 2009a). Each flavouring substance is attributed a FLAVIS-number (FL-number) and all substances are divided into 34 chemical groups. Substances within a group should have some metabolic and biological behaviour in common.

Substances which are listed in the Register are to be evaluated according to the evaluation programme laid down in Commission Regulation (EC) No 1565/2000 (EC, 2000a), which is broadly based on the Opinion of the Scientific Committee on Food (SCF, 1999a). For the submission of data by the manufacturer, deadlines have been established by Commission Regulation (EC) No 622/2002 (EC, 2002b).

After the completion of the evaluation programme the Union list of flavouring substances for use in or on foods in the EU shall be adopted (Article 5 (1) of Regulation (EC) No 2232/96) (EC, 1996a).

Flavouring Group Evaluation 19 (FGE.19) contains 360 flavouring substances from the EU Register being α,β-unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation (EFSA, 2008b).

The α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity. The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

The α,β-unsaturated carbonyls were subdivided into 28 subgroups on the basis of structural similarity (EFSA, 2008b). In an attempt to decide which of the substances could go through the Procedure, a (quantitative) structure-activity relationship (Q)SAR prediction of the genotoxicity of these substances was undertaken considering a number of models (DEREKfW, TOPKAT, DTU-NFI-MultiCASE Models and ISS-Local Models (Gry et al., 2007)).

The Panel noted that for most of these models internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for these α,β-unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the Procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 2007a; Benigni and Netzeva, 2007b) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. The Panel decided that 11 subgroups (1.1.2, 1.1.3, 1.1.4, 2.4, 2.6, 2.7, 3.1, 3.3, 4.1, 4.2 and 4.4) (EFSA, 2008b) should be further examined to determine whether evaluation through the Procedure is feasible. Corresponding to these 11 subgroups, 11 Flavouring Group Evaluations (FGEs) were established, FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220. If the Panel concludes for any substances in these 11 FGEs that they cannot be evaluated using the Procedure then it has to be decided if there is a safety concern for certain substances or if additional data are required in order to finalise the evaluation. If the Panel concludes that a genotoxic potential can be ruled out for the substances they will be merged with structurally related substances in other FGEs and evaluated using the Procedure.

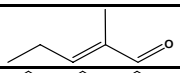
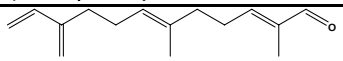
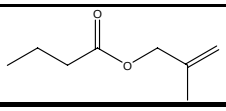
HISTORY OF EVALUATION

In FGE.201 the Panel concluded that additional genotoxicity data were required for all 11 α,β-unsaturated aldehydes and alcohols and related esters considered in the FGE.

FGE	Adopted by EFSA	Link	No. of Substances
FGE.201	25 September 2008	http://www.efsa.europa.eu/en/efsajournal/pub/1080.htm	11
FGE.201Rev1			11

In the EFSA Opinion "List of α,β-unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing" (EFSA, 2008bc), representative flavouring substances have been selected for subgroup 1.1.2, corresponding to FGE.201, for which additional data on genotoxicity were requested, according to the Opinion of the Panel on the "Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19" (EFSA, 2008bb).

Representative substances for subgroup 1.1.2 of FGE.19 (EFSA, 2008bc)

FL-no JECFA-no	EU Register name	Structural formula	Comments
05.090 1209	2-Methylpent-2-enal		
05.178 1227	beta-Sinensal		
Not in EU Register	2-methyl-2-propenal or its precursor [09.177]		
09.177 1207	2-Methylallyl butyrate		

The present FGE.201 Revision 1 (FGE.201Rev1) includes the assessment of additional genotoxicity data submitted by Industry in reply to a data request presented in FGE.201.

Additional data has been provided by Industry (EFFA, 2011g) for the representative substance 2-methylpent-2-enal [FL-no: 05.090] and a non-representative substance, also from subgroup 1.1.2, 2-methylcrotonaldehyde [FL-no: 05.095]. No data has been submitted for the other representatives identified by EFSA (EFSA, 2008bc), beta-sinensal [FL-no: 05.178], 2-methyl-2-propenal [not a Register substance but a precursor for such] or its precursor 2-methylallyl butyrate [FL-no: 09.177]. According to Industry, [FL-no: 05.178 and 09.177] are not any longer supported by the Industry and accordingly no data were submitted for these substances due to the lack of test material for the required genotoxicity testing.

The new data submitted for [FL-no: 05.090 and 05.095] are described and evaluated in Section 4 in the present FGE.201Rev1. Sections 1-3 report the same information that was present in the earlier version of FGE.201.

TERMS OF REFERENCE AS PROVIDED BY THE COMMISSION

European Food Safety Authority (EFSA) is requested to carry out a risk assessment on flavouring substances prior to their authorisation and inclusion in a Union list according to Commission Regulation (EC) No 1565/2000 (EC, 2000a).

In addition, in letter of 22 September 2011, the Commission requested EFSA to carry out a re-evaluation on the nine substances 2-methylbut-2-en-1-ol [FL-no: 02.174], 2-ethylhept-2-enal [FL-no: 05.033], 2-methylpent-2-enal [FL-no: 05.090], 2-methylcrotonaldehyde [FL-no: 05.095], 2-butylbut-2-enal [FL-no: 05.105], 2-isopropyl-5-methylhex-2-enal [FL-no: 05.107], 2-methyloct-2-enal [FL-no: 05.126], alpha-sinensal [FL-no: 05.130] and 2,6-dimethyl-2,5,7-octatriene-1-ol acetate [FL-no: 09.931], in accordance with Commission Regulation (EC) No 1565/2000 (EC, 2000a).

ASSESSMENT

1. Presentation of the Substances in the Flavouring Group Evaluation 201, Revision 1

1.1. Description

The present Flavouring Group Evaluation 201, Revision 1 (FGE.201Rev1), concerns 11 substances, which are presented in Table 1. The 11 substances correspond to subgroup 1.1.2 of FGE.19 (EFSA, 2008b). Eight of these substances are aliphatic acyclic 2-alkylated α,β-unsaturated aldehydes with or without additional double bonds [FL-no: 05.033, 05.090, 05.095, 05.105, 05.107, 05.126, 05.130 and 05.178] and three are precursors for such aldehydes [FL-no: 02.174, 09.177 and 09.931].

A summary of their current evaluation status by the JECFA is given in Table 2 (JECFA, 2004b; JECFA, 2007c).

The α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008b). Accordingly the available data on genotoxic or carcinogenic activity for the eight aldehydes in FGE.201 [FL-no: 05.033, 05.090, 05.095, 05.105, 05.107, 05.126, 05.130 and 05.178] and the two aldehydes [non-Register substances, 2,6-dimethyl-2,5,7-octatrienal and 2-methyl-2-propenal, see Table 3] anticipated to be metabolism products formed from two of the three precursors in FGE.201 [FL-no: 02.174, 09.177 and 09.931], will be considered in this FGE. The anticipated metabolism product formed from the third precursor [FL-no: 02.174] is one of the eight Register aldehydes in this FGE [FL-no: 05.095].

The Panel has also taken into consideration the outcome of the predictions from five selected (Q)SAR models (Benigni and Netzeva, 2007a; Gry et al., 2007; Nikolov et al., 2007) on the 10 aldehydes [FL-no: 05.033, 05.090, 05.095, 05.105, 05.107, 05.126, 05.130, 05.178, 2-methyl-2-propenal and 2,6-dimethyl-2,5,7-octatrienal]. The 10 aldehydes and their (Q)SAR predictions are shown in Table 3.

2. Toxicity

2.1. (Q)SAR Predictions

In Table 3 the outcomes of the (Q)SAR predictions for possible genotoxicity activity in five *in vitro* (Q)SAR models (ISS-Local Model-Ames Test, DTU-NFI-MultiCASE-Ames test, -Chromosomal aberration test in Chinese hamster ovary cells (CHO), -Chromosomal aberration test in Chinese hamster lung cells (CHL) and -Mouse Lymphoma Test) are presented. For the three short-chain aldehydes [FL-no: 05.095, 05.090 and 05.105] the predictions in the ISS Local Ames test (TA100) were positive. For 2-methyl-2-propenal, the DTU-NFI-MultiCASE Ames test was positive. All other predictions were either negative or out of domain (See Table 3).

2.2. Carcinogenicity Studies

No carcinogenicity studies are available for the eight α,β-unsaturated aldehydes and the α,β-unsaturated aldehydes anticipated to be formed from the three precursors [FL-no: 02.174, 09.177 and 09.931] in subgroup 1.1.2.

2.3. Genotoxicity Studies[4]

Only one study on 2-methylpent-2-enal [FL-no: 05.090] and one study on 2-methyl-2-propenal [not in Register] is available for the eight aldehydes and the α,β-unsaturated aldehydes anticipated to be formed from the precursors in subgroup 1.1.2. The study on 2-methylpent-2-enal is a spot test (Florin et al., 1980), which is not in accordance with the OECD guideline. Furthermore, the methods and results are insufficiently reported and the study is considered to be of insufficient validity. The study on 2-methyl-2-propenal (and the structurally related 2-propyl and 2-butyl substituted 2-propenals), in Ames test in *Salmonella typhimurium* strain TA100, showed mutagenic effects of all the 2-alkylated 2-propenals (Eder & Deininger, 2001). The study was found valid. See Table 5.

2.4. Conclusion on Genotoxicity[4]

The genotoxicity concern with respect to this group of substances due to the presence of an α,β-unsaturated aldehyde group (or precursor for this) cannot be ruled out based on the genotoxicity data and (Q)SAR predictions available.

3. Conclusion[4]

The Panel concluded that a genotoxic potential of the 11 α,β-unsaturated aldehydes and alcohols and related esters in the present FGE.201 could not be ruled out. Accordingly the 11 substances cannot be evaluated through the Procedure. Additional data on genotoxicity on substances representative for this subgroup should be provided according to the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008bb).

4. Data submitted from Industry in reply to genotoxicity data requested in FGE.201

4.1. *In vitro* assays

4.1.1. Ames tests

2-Methylpent-2-enal [FL-no: 05.090] was tested in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 in the presence and absence of metabolic activation by S-9 (Bowen, 2011). In the first experiment performed using plate incorporation methodology, concentrations of 1.6, 8, 40, 200, 1000 and 5000 µg/plate were assessed for all tester strains. Evidence of toxicity was only observed in strains TA1537 at 5000 µg/plate in the presence of S-9 and TA102 at 1000 µg/plate and above in the presence of S-9. The concentration range was therefore narrowed for a second experiment. In addition, the second experiment included a supplementary S-9 pre-incubation step for the S-9 treatment group to increase the range of assay detection. Following these treatments, evidence of toxicity was observed in the presence of S-9 in *S. typhimurium* strains TA98, TA100, TA1535 and TA102 at 2500 µg/plate and above and in strain TA1537 at 1250 µg/plate and above. Toxicity was not observed in the absence of S-9. In the second experiment, at a single intermediate dose of 2500 µg/plate without S-9 treatment in strain TA1537, there was a small (2.1-fold), statistically significant increase in revertants, but this was not dose-related and was within the range of historical controls. The Panel concluded that 2-methylpent-2-enal was not mutagenic in this study (Table 5 and 7b).

[4] The conclusions in Section 2.3, 2.4 and Section 3 are cited from the first version of the present FGE, FGE.201. This conclusion is the basis for the request of additional genotoxicity data in FGE.201.

A bacterial reverse mutation assay was also conducted in *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* strain WP2uvrA with 2-methylcrotonaldehyde [FL-no: 05.095] (Nakajima, 2006a), which does not belong to the substances selected as representative by the Panel for this subgroup. In an initial experiment the concentrations used were 8.19, 20.5, 51.2, 128, 320, 800, 2000 and 5000 µg/plate. In strain TA100, 2-methylcrotonaldehyde increased the number of colonies showing reverse mutations, both with and without metabolic activation by S-9, in a dose-dependent manner. In the absence of S-9, the increases were 3-fold from 320 - 5000 µg/plate and with S-9 metabolic activation the increases were up to 3.9-fold from 320 - 2000 µg/plate; under these conditions, growth inhibition was observed at 5000 µg/plate. In all other strains in the presence and absence of metabolic activation treatment with 2-methylcrotonaldehyde did not result in an increase of reverse mutant colony (Table 5 and 7a).

In a second experiment using 2-methylcrotonaldehyde [FL-no: 05.095] concentrations of 156, 313, 625, 1250, 2500 and 5000 µg/plate, treatment of strain TA100 in the absence of S-9 activation induced a dose-dependent increase in reverse mutant colonies (1.5 - 7.2 fold), with growth inhibition observed at 5000 µg/plate in the absence of S-9. In the presence of S-9, treatment of strain TA100 also gave a dose-dependent (313-2500 µg/plate) increase in reverse mutant colonies (1.2 - 4.6 fold), with growth inhibition again observed at 5000 µg/plate. In the absence of S-9, strain TA1535 gave a 1.8-fold increase in reverse mutants at 2500 µg/plate, but this was not dose-dependent. Strain TA98 had a nearly 2-fold increase in reverse mutant colonies in the absence of S-9 at both 2500 and 5000 µg/plate. These increases were above the historical control range but they were not clearly dose-related. In the presence of S-9, the same strain showed a 1.5-fold increase at 5000 µg/plate. *E. coli* strain WP2*uvrA* showed a 2-fold increase in reverse mutant colonies at 5000 µg/plate treatment without S-9 which was above the historical control range, but otherwise there was no increase at any other dose or with S-9 treatment (Table 5 and 7a).

Strain WP2*uvrA* did not show evidence of mutagenicity in the first experiment but produced weakly mutagenic results in the second experiment at the highest concentration tested. Since the repeatability of the result was not confirmed an additional experiment with this strain was performed from 156-5000 µg/plate and this resulted in a 1.4-fold increase in revertant colonies at 2500 and 5000 µg/plate. The Panel considered these small increases as indication for a weak mutagenic potential because the effects were reproducible in two out of three experiments and were above the historical control range. However, with the exception of strain TA100, there were no increases in reverse mutation colonies exceeding that of the negative control by 2-fold. The Panel considered that 2-methylcrotonaldehyde [FL-no: 05.095] was mutagenic in the TA100 strain in the absence and presence of S-9 under the specified conditions of the assay.

In order to clarify the ability of 2-methylcrotonaldehyde [FL-no: 05.095] to induce reverse mutations and provide cross-lab comparison of results, a third Ames assay was conducted (Ballantyne, 2011) in the same laboratory in which the Bowen 2011 study was performed. In order to directly compare 2-methylcrotonaldehyde with 2-methylpent-2-enal, the latter was tested additionally in this assay. Both substances were tested in the single *S. typhimurium* strain TA100, in the absence and the presence of metabolic activation, in three separate experiments.

The first experiment was conducted using final concentrations of 2-methylcrotonaldehyde [FL-no: 05.095] and 2-methylpent-2-enal [FL-no: 05.090] at 1.6, 8, 40, 200, 1000 and 5000 µg/plate in strain TA100 with or without S-9. Following these treatments, no evidence of toxicity was observed. Statistically significant ($p \leq 0.01$) increases in revertants were observed for 2-methylcrotonaldehyde at 5000 µg/plate in the absence of S-9 metabolic activation (1.3-fold) and at all concentrations tested in the presence of S-9. In all cases, the increases were small versus concurrent controls (1.2–1.7 fold versus concurrent controls) but were above the range of historical controls. Similarly, for 2-methylpent-2-enal, small statistically significant ($p \leq 0.05$) increases in revertants (1.2-fold) were observed at 5000 µg/plate in the absence of S-9 metabolic activation and at 1.6, 200 and 5000 µg/plate ($p \leq 0.01$) in the presence of S-9. In all cases, the increases were small versus concurrent controls

(1.2–1.4 fold versus concurrent controls) but the results obtained in the presence of S-9 were above the range of historical controls and dose-related (Table 5 and 7c).

In the second experiment, treatment of strain TA100 with 2-methylcrotonaldehyde [FL-no: 05.095] and 2-methylpent-2-enal [FL-no: 05.090] was performed in the absence and in the presence of S-9 at narrowed concentration intervals. 2-Methylcrotonaldehyde and 2-methylpent-2-enal were assayed at 51.2, 128, 320, 800, 2000 and 5000 µg/plate in the absence of S-9 using plate incorporation methodology and 8.192, 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate in the presence of S-9 using both plate incorporation and pre-incubation methodology. Clear evidence of toxicity was only observed following pre-incubation methodology treatments with 2-methylcrotonaldehyde in the presence of S-9 at 5000 µg/plate and pre-incubation methodology treatments with 2-methylpent-2-enal in the presence of S-9 at 2000 and 5000 µg/plate. In the absence of S-9 activation, small but statistically significant increases in revertant colonies showing a dose-dependent relationship were observed for 2-methylcrotonaldehyde at 800, 2000 and 5000 µg/plate. These were only 1.3-1.4 fold over concurrent vehicle controls but above the range of historical controls. In the presence of S-9 activation, 2-methylcrotonaldehyde showed a small but statistically significant increase in revertant colonies only at 5000 µg/plate using plate incorporation (1.4-fold) and at 800 and 2000 µg/plate using pre-incubation methodology (1.7-fold). These increases were above the historical control range. Similarly, in the presence of S-9 activation 2-methylpent-2-enal showed a small (1.4-fold) but statistically significant increase in revertants only at 5000 µg/plate using plate incorporation methodology, but in contrast gave no increases in revertant colonies using pre-incubation. In the absence of S-9 activation, a statistically significant increase in revertant colonies was observed at 5000 µg/plate that was 2.5-fold over concurrent vehicle controls. These increases were above the historical control range, likewise (Table 5 and 7c).

In order to further investigate the reproducibility and dose-relationship of some increases in revertant numbers seen in the first two experiments, a third experiment was performed with 2-methylcrotonaldehyde [FL-no: 05.095] in the presence of S-9 (plate incorporation methodology only) and with 2-methylpent-2-enal in the absence of S-9. In order to investigate the dose range over which the increases in revertant numbers were previously observed, treatment concentration ranges of 0.32 - 5000 µg/plate and 51.2 - 5000 µg/plate were employed for 2-methylcrotonaldehyde and 2-methylpent-2-enal, respectively. Following these treatments, no evidence of toxicity was observed with both substances. For 2-methylcrotonaldehyde, small (1.3- to 1.8-fold) but statistically significant and dose-related increases in revertants were observed at 1000 and 5000 µg/plate. These increases were above the historical control range. For 2-methylpent-2-enal, a small but statistically significant increase in revertants was observed at 5000 µg/plate. This increase was only 1.3-fold versus concurrent vehicle controls but above the historical control range.

Overall, statistically significant increases in revertant numbers (when the data were analysed at the 1 % level using Dunnett's test) were observed in the absence and presence of S-9 in each experiment where the TA100 strain was treated with 2-methylcrotonaldehyde [FL-no: 05.095]. These increases provided evidence of a dose-relationship, with the exception of the first experiment in the absence of S-9, where the largest increase was observed at the lowest treatment concentration (1.6 µg/plate) and was attributed to an aberrant occurrence that was not reproduced in subsequent experiments. While the magnitude of these increases were small (1.3 - 1.8-fold above the concurrent control levels), these data were considered by the authors of the study report as evidence of 2-methylcrotonaldehyde mutagenic activity in strain TA100 in the absence and presence of metabolic activation with S-9. The Panel noted that these increases, although being small, was above the range of historical controls and that these effects were generally reproducible in the different experiments of this study. Thus, the Panel concluded that this study provided an indication for a weak mutagenic activity of 2-methylcrotonaldehyde [FL-no: 05.095] in the presence and absence of metabolic activation.

2-Methylpent-2-enal [FL-no: 05.090] treatments of strain TA100 in the absence and presence of S-9, assayed simultaneously for comparison to 2-methylcrotonaldehyde, resulted in statistically significant increases in revertant numbers in the absence and presence of S-9 in each experiment, with the

exception of experiment 2 using pre-incubation methodology in the presence of S-9. The maximum treatment concentration in the experiment 2 in the absence of S-9 resulted in a statistically significant increase of 2.5-fold over the concurrent control level, though this was not reproduced in experiments 1 or 3, nor previously observed (Bowen, 2011). However, the small increases observed in experiments 1 and 3 were statistically significant and above the range of historical controls. Thus, there is at least some consistency in these three experiments. The authors of the study report (Ballantyne, 2011) noted that all of the observed increases provided at least some evidence of dose dependence (in most cases the only responding concentration being the highest treatment concentration), and were reproducible over most of the treatment occasions. Accordingly, the authors considered that these increases are indicative of weak 2-methylpent-2-enal mutagenic activity in strain TA100 in the absence and in the presence of S-9 in this assay system. The Panel agreed with the authors.

The details and conclusions for the Ames tests described above are summarised in Tables 5 and 7a to 7d.

4.1.2. In vitro Micronucleus assay

2-Methylpent-2-enal [FL-no: 05.090] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat liver metabolic activation system (S-9) as an *in vitro* metabolising system (Whitwell, 2011a). Cells were stimulated for 48 hours with phytohaemagglutinin to produce exponential cell growth followed by treatment for 3 hours (with a 21 hours recovery period) in the presence or absence of S-9 or 24 hours treatment with no recovery in the absence of S-9. In the first experiment, doses of 0, 100, 200 and 300 µg/ml of 2-methylpent-2-enal were tested. Frequencies of micronucleated binucleate (MNBN) cells were not significantly different ($p \leq 0.05$) from that of the negative control in all conditions with the exception of the highest concentration analyzed post 3 + 21 hours without S-9 treatment (300.0 µg/ml, inducing 49 % cytotoxicity, mean MNBN cells 1.05 %). This increase, although statistically significant relative to the concurrent vehicle control, was relatively small and did not exceed the range of historical controls (0.1 to 1.2 % MNBN cells). Furthermore, both replicate cultures at this concentration and all other 2-methylpent-2-enal concentrations analyzed were within a normal range of MNBN cell values.

To clarify these data, a 3 + 21 hours treatment in the absence of S-9 was performed in a second experiment with 100.0, 175.0, 260.0 and 300.0 µg/ml concentrations of 2-methylpent-2-enal. Statistically significant increases in micronucleated cell frequencies were not observed. Consistent with current regulatory guideline recommendations for this assay, the maximum concentrations that were analyzed induced between 49 % to 57 % cytotoxicity. The Panel agreed with the authors of the study report and concluded that 2-methylpent-2-enal did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of metabolic activation with S-9 (Table 5).

4.1.3. In vitro chromosomal aberration assay

The ability of 2-methylcrotonaldehyde [FL-no: 05.095] to induce chromosomal aberrations was evaluated in an *in vitro* assay using Chinese hamster pulmonic fibroblasts (Nakajima, 2006b). Microscopic observations were conducted during the chromosome aberration tests using short-term treatment with 3 or 4 different doses respectively, 105, 210 and 421 µg/ml in the absence of S-9 and 105, 210, 421 and 841 µg/ml in the presence of S-9. The frequencies of chromosome structural aberrations as a result of short-term treatment of 2-methylcrotonaldehyde without metabolic activation by S-9 were 0.5 % in the negative control, 3.5 % in the 105 µg/mL treatment, 12.0 % in the 210 µg/mL treatment, 55 % in the 421 µg/ml treatment and 50.0 % in the mitomycin positive control. The appearance of polyploidy cells was not observed at any dose and there were no significant reductions in relative cell growth rate under these test conditions. In the presence of S-9, the frequencies of chromosome structural aberration as a result of treatment using 2-methylcrotonaldehyde were 0.05 %

in the negative control, 1.5 % in the 105 µg/ml dose, 1.5 % in the 210 µg/ml dose, 33.0 % in the 421 µg/ml dose, 96.5 % in the 841 µg/ml dose and 39 % in the cyclophosphamide positive control treatment group. The frequencies of appearance of polyploidy cells were equivalent to those of the negative control group at all doses and there was no significant reduction in relative cell growth rate. Therefore, a dose-dependent induction of structural chromosome aberrations was associated with 2-methylcrotonaldehyde treatment in both short-term treatments with and without S-9 under testing conditions. An increase in numerical chromosomal aberrations was not observed. The Panel agreed with the authors of the study report and concluded that 2-methylcrotonaldehyde induced chromosomal aberrations in cultured mammalian cells in the presence and absence of metabolic activation (Table 5).

4.2. *In vivo* genotoxicity tests

4.2.1. Micronucleus assay

2-Methylcrotonaldehyde [FL-no: 05.095] was tested in an *in vivo* micronucleus assay using BDF_1 male mice (Nakajima, 2007). Five mice per group were administered by oral gavage with a dose of 2-methylcrotonaldehyde once per day for two consecutive days at either 250, 500 or 1000 mg/kg bw (1000 mg/kg bw was the maximum tolerable dose based on an initial dose-finding study in which two out of three animals died after administration of 2000 mg/kg bw). The bone marrow cells were sampled 24 hours after the second dosing. Administration of 2-methylcrotonaldehyde resulted in one case of pilo-erection 25 hours after the first administration of 1000 mg/kg. No other treatment groups showed any sign of toxicity by means of visual examination or by body weight loss. There was no statistically significant increase in the frequency of micronucleated erythrocytes in treated groups compared to the negative control group. The ratio of polychromatic erythrocytes among total erythrocytes was not changed. Thus, the exposure of the bone marrow to the test substance could not be demonstrated based on that parameter. However, since mortality was observed at a dose of 2000 mg/kg bw in the dose-finding study the Panel considered that systemic availability of the test substance could be assumed at the highest dose of 1000 mg/kg bw in the micronucleus assay. Although, it may be assumed that the bone marrow was exposed to the test substance since the bone marrow is a well perfused tissue, no conviencing evidence was provided. The Panel considered the study to be compliant with OECD guideline 474 except that no justification for the use of a single sex was given in the report, i.e. no data demonstrating that there are no substantial differences between sexes in toxicity. It was concluded that 2-methylcrotonaldehyde did not induce micronuclei in mice bone-marrow cells (Table 6).

5. Discussion of the additional data

2-Methylpent-2-enal [FL-no: 05.090] and 2-methylcrotonaldehyde [FL-no: 05.095] were tested in a series of *in vitro* tests to explore their genotoxicity potential.

For 2-methylcrotonaldehyde [FL-no: 05.095], there is some evidence for a mutagenic activity in the presence and absence of metabolic activation. This is based on two reliable studies on the induction of bacterial gene mutations (Nakajima, 2006a; Ballantyne, 2011) with the first study providing clear evidence for mutagenic activity and the latter study providing indication for a weak mutagenic activity.

For the representative substance 2-methylpent-2-enal [FL-no: 05.090] there is an indication for a weak mutagenic activity both in the presence and absence of metabolic activation. This indication is based on the results of one reliable study (Ballantyne, 2011) and structural similarity with 2-methylcrotonaldehyde [FL-no: 05.095].

In vitro assessment of micronucleus induction by 2-methylpent-2-enal in the presence and absence of S-9 metabolic activation was negative. In the case of 2-methylcrotonaldehyde a dose-dependent induction of structural, but not numerical chromosomal aberrations *in vitro* was observed, both with and without S-9. To further investigate the clastogenic potential an *in vivo* micronucleus assay using BDF_1 male mice was performed and no evidence of clastogenic effects was identified but the

respective test was inconclusive. These data support absence of clastogenicity for the two substances 2-methylpent-2-enal [FL-no: 05.090] and 2-methylcrotonaldehyde [FL-no: 05.095].

However, since there is some evidence for 2-methylcrotonaldehyde [FL-no: 05.095] and an indication for 2-methylpent-2-enal [FL-no: 05.090] to show a potency for the induction of gene mutations *in vitro* the Panel considered the mutagenicity hazard not cleared by the endpoints evaluated in the *in vivo* micronucleus assay. The Panel therefore concluded that further data are required in order to clarify the genotoxic potential of this subgroup. The Panel considers the *in vivo* Comet assay as a preferred option to further investigate the genotoxicity *in vivo*. The Comet assay will also be accountable to explore any genotoxicity at the first site of contact where higher concentrations of the test substance are expected to occur. In this view the *in vivo* Comet assay should be performed on liver, blood and first site of contact (e.g. duodenum or stomach). Alternatively, a transgenic rodent gene mutation assay (OECD TG 488) in tissues including first site of contact would also be acceptable.

Since 2-methylpent-2-enal [FL-no: 05.090] and 2-methylcrotonaldehyde [FL-no: 05.095] are closely related chemical structures, it is expected that they will have a similar reactivity behaviour. Since the evidence for a mutagenic activity *in vitro* is stronger for 2-methylcrotonaldehyde [FL-no: 05.095] than for 2-methylpent-2-enal [FL-no: 05.090] the Panel considers that additional data for the former substance are required. A negative result of 2-methylcrotonaldehyde [FL-no: 05.095] in the *in vivo* assay would be considered representative for the following substances of this subgroup [FL-no: 02.174, 05.033, 05.090, 05.105, 05.107 and 05.126]. A positive result of 2-methylcrotonaldehyde (FL-no: 05.095) in the *in vivo* assay will require further testing of these six substances [FL-no: 02.174, 05.033, 05.090, 05.105, 05.107 and 05.126] in order to finalise their evaluation.

The Panel noted that the Industry has communicated that the following two substances [FL-no: 05.178 and 09.177] are not supported any more. These two substances were selected as representative for the substances in subgroup 1.1.2 and in addition also for substances in subgroup 2.1 (FGE.207). Since no data will be provided for these substances, they cannot further be used as representatives for the substances [FL-no: 02.122, 09.034, 09.712, 09.809] in FGE.207.

2-Methylpent-2-enal (FL-no: 05.090) was originally selected as representative for the subgroup 1.1.2 and also for the substances in subgroup 5.3 (FGE.225). Since the Panel now considers that additional data are required for 2-methylcrotonaldehyde these data could also be considered representative for the substances in FGE.225 [FL-no: 12.065, 12.079].

The genotoxicity studies are summarized in Tables 5, 6 and 7a to 7d.

6. Conclusion

Since there is some evidence for 2-methylcrotonaldehyde [FL-no: 05.095] and an indication for 2-methylpent-2-enal [FL-no: 05.090] to show a potency for the induction of gene mutations *in vitro* the Panel considered the mutagenicity hazard not cleared by the endpoints evaluated in the *in vivo* micronucleus assay on 2-methylcrotonaldehyde [FL-no: 05.095]. The Panel therefore concluded that further data are required in order to clarify the genotoxic potential of this subgroup. The Panel considers the *in vivo* Comet assay as a preferred option to further investigate the genotoxicity *in vivo*. Since the evidence for a mutagenic activity *in vitro* is stronger for 2-methylcrotonaldehyde [FL-no: 05.095] than for 2-methylpent-2-enal [FL-no: 05.090] the Panel considers that additional data for 2-methylcrotonaldehyde are required. The Panel concluded that the genotoxicity data for 2-methylcrotonaldehyde cannot be considered representative for the remaining substances of this subgroup [FL-no: 05.130, 05.178, 09.177 and 09.931] for which it was already concluded in the previous version of this FGE that the available data were insufficient to evaluate their genotoxicity.

The Panel noted that this conclusion will also have consequences for the read across for substances in subgroups 2.1 and 5.3 (FGE.207 and FGE.225).

TABLE 1 SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FGE.201REV1

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 201, Revison 1 (JECFA, 2003b)

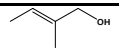
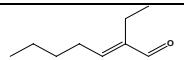
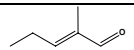
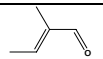
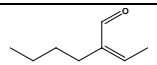
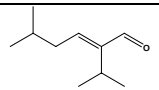
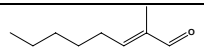
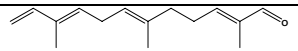
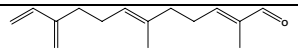
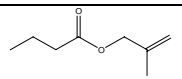
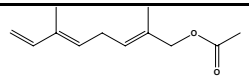
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
02.174 1617	2-Methylbut-2-en-1-ol		 10258 4675-87-0	Liquid $C_5H_{10}O$ 86.13	 Freely soluble	137 95 %	1.439-1.445 0.863-0.869
05.033 1216	2-Ethylhept-2-enal		2438 120 10031-88-6	Liquid $C_9H_{16}O$ 140.23	Insoluble Soluble	55-60 (5 hPa) NMR 97 %	1.460-1.466 0.891-0.898
05.090 1209	2-Methylpent-2-enal		3194 2129 623-36-9	Liquid $C_6H_{10}O$ 98.15	Insoluble Soluble	137 IR MS 92 %	1.445-1.453 0.855-0.865
05.095 1201	2-Methylcrotonaldehyde		3407 2281 497-03-0	Liquid C_5H_8O 84.12	Slightly soluble Soluble	117-118 IR NMR 99 %	1.445-1.450 0.868-0.873 (20°)
05.105 1214	2-Butylbut-2-enal		3392 10324 25409-08-9	Liquid $C_8H_{14}O$ 126.20	Insoluble Soluble	50 (18 hPa) NMR 97 %	1.447-1.453 1.449-1.459 (20°)
05.107 1215	2-Isopropyl-5-methylhex-2-enal		3406 10361 35158-25-9	Liquid $C_{10}H_{18}O$ 154.25	Insoluble Soluble	189 NMR 95 %	1.448-1.454 0.840-0.846
05.126 1217	2-Methyloct-2-enal		3711 10363 49576-57-0	Liquid $C_9H_{16}O$ 140.23	Insoluble Soluble	70-75 (10 hPa) IR 96 %	1.449-1.459 0.872-0.882
05.130	alpha-Sinensal		3141 10380 17909-77-2	 218.34			
05.178 1227	beta-Sinensal		3141 10381 60066-88-8	Liquid $C_{15}H_{22}O$ 218.34	Insoluble Soluble	180 (1 hPa) NMR 99 %	1.504-1.513 0.917-0.923
09.177 1207	2-Methylallyl butyrate		2678 572 7149-29-3	Liquid $C_8H_{14}O_2$ 142.20	Insoluble Soluble	168 NMR 97 %	1.422-1.428 0.873-0.883

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 201, Revison 1 (JECFA, 2003b)

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
09.931 1226	2,6-Dimethyl-2,5,7-octatriene-1-ol acetate		3886 999999-91-4	Liquid $C_{12}H_{18}O_2$ 194.28	Insoluble Soluble	70 (3 hPa) IR NMR MS 96 %	1.490-1.500 0.937-0.947

1) Solubility in water, if not otherwise stated.

2) Solubility in 95 % ethanol, if not otherwise stated.

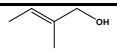
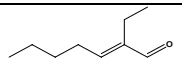
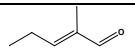
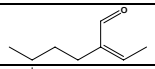
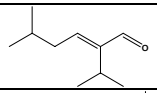
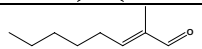
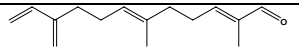
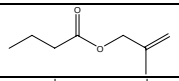
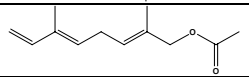
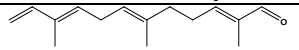
3) At 1013.25 hPa, if not otherwise stated.

4) At 20°C, if not otherwise stated.

5) At 25°C, if not otherwise stated.

TABLE 2 SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach) (JECFA, 2004b; JECFA, 2007c)

FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) (µg/capita/day) EU USA	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5]	EFSA conclusion
02.174 1617	2-Methylbut-2-en-1-ol		0.037	Class I A3: Intake below threshold	4)	Evaluated in FGE.201Rev1, additional *in vivo* genotoxicity data required
05.033 1216	2-Ethylhept-2-enal		0.012 0.1	Class I A3: Intake below threshold	4)	Evaluated in FGE.201Rev1, additional *in vivo* genotoxicity data required
05.090 1209	2-Methylpent-2-enal		3.4 0.2	Class I A3: Intake below threshold	4)	Evaluated in FGE.201Rev1, additional *in vivo* genotoxicity data required
05.095 1201	2-Methylcrotonaldehyde		0.61 0.2	Class I A3: Intake below threshold	4)	Evaluated in FGE.201Rev1, additional *in vivo* genotoxicity data required
05.105 1214	2-Butylbut-2-enal		0.0 0.01	Class I A3: Intake below threshold	4)	Evaluated in FGE.201Rev1, additional *in vivo* genotoxicity data required
05.107 1215	2-Isopropyl-5-methylhex-2-enal		0.24 0.01	Class I A3: Intake below threshold	4)	Evaluated in FGE.201Rev1, additional *in vivo* genotoxicity data required
05.126 1217	2-Methyloct-2-enal		0.0 7.9	Class I A3: Intake below threshold	4)	Evaluated in FGE.201Rev1, additional *in vivo* genotoxicity data required
05.178 1227	beta-Sinensal		0.91 0.5	Class I A3: Intake below threshold	4)	Evaluated in FGE.201, additional genotoxicity data required
09.177 1207	2-Methylallyl butyrate		ND 0.2	Class I A3: Intake below threshold	4)	Evaluated in FGE.201, additional genotoxicity data required
09.931 1226	2,6-Dimethyl-2,5,7-octatriene-1-ol acetate		1.2 7.7	Class I A3: Intake below threshold	4)	Evaluated in FGE.201, additional genotoxicity data required
05.130	alpha-Sinensal			Not evaluated by JECFA		Evaluated in FGE.201, additional genotoxicity data required

1) EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.

2) Thresholds of concern: Class I = 1800 µg/person/day , Class II = 540 µg/person/day, Class III = 90 µg/person/day.

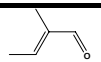
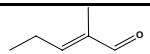
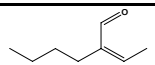
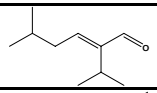
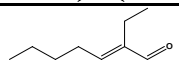
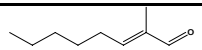
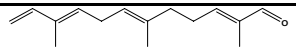
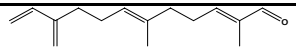
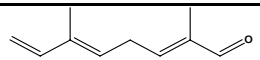
3) Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.

4) No safety concern based on intake calculated by the MSDI approach of the named compound.

5) Data must be available on the substance or closely related substances to perform a safety evaluation.

TABLE 3 (Q)SAR PREDICTIONS ON MUTAGENICITY FOR NINE ALDEHYDES REPRESENTING THE SUBSTANCES IN SUBGROUP 1.1.2

Table 3: (Q)SAR Predictions on Mutagenicity in Five Models for nine aldehydes Representing the Substances in Subgroup 1.1.2

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	ISS Local Model Ames Test TA100	MultiCASE Ames test	MultiCASE Mouse lymphoma test	MultiCASE Chromosmal aberration test in CHO	MultiCASE Chromosmal aberration test in CHL
05.095 1201	2-Methylcrotonaldehyde		3407 2281 497-03-0	POS	NEG	OD	OD	NEG
05.090 1209	2-Methylpent-2-enal		3194 2129 623-36-9	POS	NEG	OD	NEG	NEG
05.105 1214	2-Butylbut-2-enal		3392 10324 25409-08-9	POS	NEG	OD	OD	NEG
05.107 1215	2-Isopropyl-5-methylhex-2-enal		3406 10361 35158-25-9	NEG	NEG	OD	OD	OD
05.033 1216	2-Ethylhept-2-enal		2438 120 10031-88-6	NEG	NEG	OD	OD	NEG
05.126 1217	2-Methyloct-2-enal		3711 10363 49576-57-0	NEG	NEG	NEG	NEG	NEG
05.130	alpha-Sinensal		3141 10380 17909-77-2	NEG	NEG	OD	NEG	NEG
05.178 1227	beta-Sinensal		3141 10381 60066-88-8	NEG	NEG	OD	NEG	NEG
Not in Register	2-methyl-2-propenal		- - 78-85-3	NYA	POS	OD	OD	OD
Not in Register	2,6-dimethyl-2,5,7-octatrienal		- - -	NYA	NEG	OD	NEG	NEG

Column 2: Structure group 1.1.2: Aliphatic acyclic α,β-unsaturated 2-alkylated aldehydes.

Column 6: Local model on aldehydes and ketones, Ames TA100. (NEG: Negative; POS: Positive; OD: out of domain; NYA: not yet assessed).

Column 7: MultiCase Ames test (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

Column 8: MultiCase Mouse Lymphona test (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

Column 9: MultiCase Chromosomal aberration in CHO (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

Column 10: MultiCase Chromosomal aberration in CHL (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

OD, out of applicability domain: not matching the range of conditions where a reliable prediction can be obtained in this model. These conditions may be physicochemical, structural, biological, etc.

TABLE 4 CARCINOGENICITY STUDIES

No carcinogenicity studies are available for the aldehydes and the aldehydes anticipated to be formed from the precursors in subgroup 1.1.2.

TABLE 5 GENOTOXICITY (*IN VITRO*)

In vitro studies available for the group of aldehydes and the aldehydes anticipated to be formed from the precursors in subgroup 1.1.2.

Table 5: Genotoxicity (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments [b)]
2-Methylpent-2-enal [05.090]	Reverse mutation	*S. typhimurium* TA98, TA100	0.03–3mmol/plate (2.94–294mg/plate)	Negative[a]	(Florin et al., 1980)	Insufficient validity (spot test, not according to OECD guideline, methods and results insufficiently reported).
		S. typhimurium TA98, TA100, TA102, TA1535 and TA1537	1.6, 8, 40, 200, 1000 and 5000 µg/plate	Negative[a,c]	(Bowen, 2011)	[d] Valid. The study was performed in compliance with GLP and according to OECD TG 471.
			78.13, 156.13, 312.5, 625, 1250, 2500 and 5000 µg/plate	Negative[a,h]		[f]
		S. typhimurium TA100	1.6, 8, 40, 200, 1000 and 5000 µg /plate	Negative (-S9, Plate)[a,c] Weakly positive (+S9, Plate)	(Ballantyne, 2011)	[g] Valid. The study was performed in compliance with GLP and according to OECD TG 471 except that only one bacterial strain was used.
			51.2, 128, 320, 800, 2000 and 5000 µg/plate	Weakly positive (-S9, Plate)[c,e]		
			8.192, 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate	Weakly positive (+S9, Plate) Negative (+S9, Pre-inc)[c, h, i]		
			51.2, 128, 320, 800, 2000 and 5000 µg/plate	Weakly positive (-S9, Plate)[c,e]		
	Micronucleus induction	Human peripheral blood lymphocytes	100, 200 and 300 µg/ml	Negative[e,j]	(Whitwell, 2011a)	[k] Valid. The study was performed in compliance with GLP and according to OECD TG 487.
			200, 275 and 350 µg/ml	Negative[i,j]		
			20, 50, 70 and 80 µg/ml 100, 175, 260 and 300	Negative[e,l] Negative[c, j]		
2-methylacrolein 2-Methyl-2-propenal	Ames test	*S. typhimurium* TA100	0-2 micromol/plate (- S9) 0-9 micromol/plate (+ S9)	Positive[a]	(Eder and Deininger, 2001)	Valid. Positive both with and without S9-mix. Toxic at 1 micromol/plate and above (- S9) and 6 micromol/plate (+ S9) evident as a reduction in revertants.
2-Methylcrotonaldehyde [05.095]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, and *Escherichia coli* WP2uvrA	8.19, 20.5, 51.2, 128, 320, 800, 2000 and 5000 µg/plate	Positive[a,c] (TA100)	(Nakajima, 2006a)	[m] Valid. According to the study report, the study was performed in compliance with Japanese GLP standards. The study report contained a certificate of reliability but no details of inspection. The study is in accordance with OECD except that only two
			156, 313, 625, 1250, 2500 and 5000 µg/plate	Positive[a,c] (TA100) Weakly positive (WP2uvrA, -S9)		
		E. coli WP2uvrA	156, 313, 625, 1250, 2500 and	Negative[a,]		

Table 5: Genotoxicity (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments [b)]
			5000 µg/plate			plates were used per concentration. No statistics were performed.
		S. typhimurium TA100	1.6, 8, 40, 200, 1000 5000 µg/plate	Weakly positive (+/- S9, Plate)[a,c]	(Ballantyne, 2011)	[n] Valid. The study was performed in compliance with GLP and according to OECD TG 471 except that only one bacterial strain was used.
			51.2, 128, 320, 800, 2000 and 5000 µg/plate	Weakly positive (+/- S9, Plate)[a,c]		
			8.192, 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate	Weakly positive (+S9, Pre)[h,i]		
			0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate	Weakly positive (+S9, Plate)[c,i]		
	Chromosomal aberration	Chinese hamster Pulmonic fibroblasts	105, 210 and 421 µg/mL without S-9 treatment and 105, 210, 421 and 841 µg/mL with S-9 treatment	Positive	(Nakajima, 2006b)	[o] Valid. According to the study report, the study was performed in compliance with Japanese GLP standards. The study report contained a certificate of reliability but no details of inspection. Mainly in accordance with OECD TG 473.

a With and without metabolic activation.

b Validity of genotoxicity studies:

Valid.

Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).

Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards inappropriate / not validated test system).

Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided, text not in a Community language).

c Plate incorporation method.

d Toxicity was observed in TA1537 at 5000 µg/plate in the presence of S-9 and in TA102 at 1000 µg/plate and above in the presence of S-9.

e Without S9 metabolic activation.

f Toxicity was observed in all strains in the presence of S-9 above 2500 µg/plate and 1250 µg/plate in TA1537. Study design complied with current recommendations. Acceptable top concentrations were achieved.

g Throughout experiments some small but statistically significant increases were seen but these were attributed to normal biological variability, and were generally less than 2-fold over concurrent vehicle controls.

h Pre-incubation method.

i With S9 metabolic activation.

j 3-hours incubation with 21-hours recovery period.

k Complies with draft OECD guideline 487. Acceptable levels of cytotoxicity were achieved at the top concentrations used in all parts of the study.

l 24-hours incubation with no recovery period.

m Study design complied with curent recommendations. Acceptable top concentrations were achieved.

n Throughout experiments some small but statistically significant increases were generally less than 2-fold over concurrent vehicle controls.

o Dose dependent increase in induction of structural chrosomal abberations with and without S-9 treatment. No changes in numerical chromosomal aberrations were observed.

TABLE 6 GENOTOXICITY (*IN VIVO*)

One *in vivo* study is available for the group of aldehydes and the aldehydes anticipated to be formed from the precursors in subgroup 1.1.2.

Table 6: Genotoxicity (*in vivo*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments b)
Trans-2-methyl-2-butenal [05.095]	*In vivo* Micronucleus induction	BDF_1 male mice	250, 500 and 1000 mg/kg bw per day by oral gavage	Negative	(Nakajima, 2007)	Valid. According to the study report, the study was performed in compliance with Japanese GLP standards. The study report contained a certificate of reliability but no details of inspection. The Panel considered the study to be compliant with OECD guideline 474 except that no justification for the use of a single sex was given in the report, i.e. no data demonstrating that there are no substantial differences between sexes in toxicity.

b Validity of genotoxicity studies:

Valid.

Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).

Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards inappropriate / not validated test system).

Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided, text not in a Community language).

TABLE 7A AMES-TEST WITH 2-METHYL-2-BUTENAL [05.095] (NAKAJIMA, 2006A)

Non-toxic effects

Table 7a: Ames-Test with 2-Methyl-2-butenal [05.095] (Nakajima, 2006a)

	S9	Assay	TA 98	TA 100	TA 1535	TA 1537	WP2*uvrA*	Comment	Conclusion
Range-finding Experiment	-	Pre	1.4-fold < HC d-r	3-fold > HC d-r	2.0-fold < HC not d-r	1.6-fold < HC not d-r	1.2-fold < HC (d-r)		Positive in TA 100
	+	Pre	1.2-fold < HC (d-r)	3.9-fold > HC d-r	1.4-fold < HC not d-r	1.0-fold < HC	1.3-fold < HC (d-r)		Positive in TA 100
Experiment 1	-	Pre	2.0-fold > HC not d-r	7.2-fold > HC d-r	1.8-fold < HC not d-r	1.9-fold < HC (d-r)	2.1-fold > HC (d-r)	WP2*uvrA*-result not clearly reproducible	Positive in TA 100, equivocal in WP2*uvrA*
	+	Pre	1.5-fold < HC (d-r)	4.6-fold > HC d-r	1.5-fold < HC not d-r	1.0-fold < HC	1.2-fold < HC not d-r		Positive in TA 100
Experiment 2	-	Pre					1.4-fold > HC (d-r)		Equivocal
	+	Pre							

Two plates were used per concentration. No examinations using statistical procedures were conducted.

> HC, above historical control; < HC, within historical control.

d-r, dose-related; (d-r), not clearly dose-related but the highest dose resulted in the largest increase, not d-r, not dose-related.

Pre, Pre-incubation assay

TABLE 7B AMES-TEST WITH 2-METHYLPENT-2-ENAL [05.090] (BOWEN, 2011)

Statistically significant increases, non-toxic effects

Table 7b: Ames-Test with 2-Methylpent-2-enal [05.090] (Bowen, 2011)

	S9	Assay	TA 98	TA 100	TA 1535	TA 1537	TA 102	Comment	Conclusion
Range-finding Experiment	-	Plate		1.2-fold < HC d-r					Negative
	+	Plate		1.2-fold < HC not d-r					Negative
Experiment 1	-	Plate	NS	NS	NS	NS	NS		Negative
	+	Plate	NS	1.2-fold < HC not d-r	NS	NS	NS		Negative
Experiment 2	-	Pre	NS	1.2-fold < HC not d-r	NS	2.1-fold < HC not d-r	NS		Negative
	+	Pre	NS	NS	NS	1.6-fold < HC not d-r	NS		Negative

> HC, above historical control; < HC, within historical control.

NS, statistically not significant.

d-r, dose-related but only the highest dose statistically significant; D-R, dose-related and at least two doses statistically significant.

TABLE 7C AMES-TEST WITH TA 100 (BALLANTYNE, 2011)

Statistically significant increases not accompanied by toxicity

Table 7c: Ames-Test with TA 100 (Ballantyne, 2011)

Register name [FL-no]	S9	Assay	Exp 1	Exp 2	Exp 3	Comment	Conclusion
2-Methylcrotonaldehyde [05.095]	-	Plate	1.3-fold > HC Not d-r	1.4-fold > HC d-r		Reproducible	Indication for a weak mutagenic activity
	+	Plate	1.7-fold > HC d-r	1.4-fold > HC d-r	1.8-fold > HC d-r	Reproducible	Indication for a weak mutagenic activity
	-	Pre					
	+	Pre		1.7-fold > HC d-r		Reproducible when compared with the plate-incorporation experiment	Indication for a weak mutagenic activity
2-Methylpent-2-enal [05.090]	-	Plate	1.2-fold < HC d-r	2.5-fold > HC d-r	1.3-fold > HC d-r	Reproducible	Indication for a weak mutagenic activity
	+	Plate	1.4-fold > HC d-r	1.4-fold > HC d-r		Reproducible	Indication for a weak mutagenic activity
	-	Pre					
	+	Pre		NS < HC			

> HC, above historical control; < HC, within historical control.

NS, statistically not significant.

d-r, dose-related but only the highest dose statistically significant; D-R, dose-related and at least two doses statistically significant.

TABLE 7D AMES-TEST RESULTS FOR 2-METHYLCROTONALDEHYDE [05.095] AND 2-METHYLPENT-2-ENAL [05.090]

Table 7d: Ames-Test results for 2-methylcrotonaldehyde [05.095] and 2-methylpent-2-enal [05.090]

Register name [FL-no]	S9	Nakajima (2006)	Bowen (2011)	Ballantyne (2011)	Comment	Conclusion
2-Methylcrotonaldehyde [05.095]	-	Positive		Indication for a weak mutagenic activity	At least the indication is reproducible.	Indication for a mutagenic activity based on two studies.
	+	Positive		Indication for a weak mutagenic activity	At least the indication is reproducible.	Indication for a mutagenic activity based on two studies.
2-Methylpent-2-enal [05.090]	-		Negative	Indication for a weak mutagenic activity	The indication was not reproducible in different studies. Inconsistent results.	Indication for a weak mutagenic activity based on the results of one study and structural similarity with 2-methylcrotonaldehyde [05.095].
	+		Negative	Indication for a weak mutagenic activity	The indication was not reproducible in different studies. Inconsistent results.	Indication for a weak mutagenic activity based on the results of one study and structural similarity with 2-methylcrotonaldehyde [05.095].

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ABBREVIATIONS

BW	Body weight
CAS	Chemical Abstract Service
CHL	Chinese hamster lung cell(s)
CHO	Chinese hamster ovary cell(s)
CoE	Council of Europe
DNA	Deoxyribonucleic acid
DTU-NFI	Danish Technical University – National Food Institute
EC	European Commission
EFSA	The European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS	Flavour Information System database
ID	Identity
IR	Infrared spectroscopy
ISS	Istituto Superiore di Sanita
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MS	Mass spectrometry
MSDI	Maximum Survey-derived Daily Intake
NMR	Nuclear magnetic resonance
No	Number
NOAEL	No observed adverse effect level
NTP	National Toxicology Programme
OECD	Organisation for Economic Co-operation and Development
PCE/NCE	Polychromatic erythrocytes/normochromatic erythrocytes
(Q)SAR	(Quantitative) structure-activity relationship
SCE	Sister chromatid exchange
SCF	Scientific Committee on Food
UDS	Unscheduled DNA synthesis
US EPA	United States Environmental Protection Agency
WHO	World Health Organisation